Adventitious Presence of Herbicide Resistant Wheat in Certified and Farm-Saved Seed Lots

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ABSTRACT

Varietal purity in wheat (*Triticum aestivum* L.) seed production is necessary for agronomic uniformity and to enable potential market segregation. We conducted a survey of certified and farm-saved seed samples using a nontransgenic imidazolinone-resistant (IR) wheat cultivar in 2004 and 2005 in eastern Colorado. The objective was to compare varietal purity based on type of seed producer and IR wheat history. Ninety-two samples of non-IR varieties were taken from certified and farm-saved seed growers, who either produced or had never produced IR wheat. Adventitious IR seeds were detected using a seed-soaking technique in samples from each producer type and each IR production history. Levels of IR seed ranged from 0 to 11.28%. One certified sample and three farm-saved samples exceeded the 0.1% threshold for off-types in certified wheat seed. Using a two-factor analysis, farm-saved production class and positive IR history increased the estimated proportion of adventitious seed. Based on grower interviews, higher levels of adventitious seed presence were associated with volunteer plants from previous crops of the resistant cultivar and mechanical mixture during harvesting. Production practices for certified seed address these factors and may need to be strengthened if more stringent purity criteria are adopted. This information is important for risk assessment and policy development for potential commercial release of transgenic wheat varieties.

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Abbreviations: ALS, acetolactate synthase; GE, genetically engineered; IR, imidazolinone resistant; PCR, polymerase chain reaction.

Gene flow in wheat (*Triticum aestivum* L.) may occur via pollen movement between fields and by adventitious, or unintentional, presence of a different variety in an otherwise pure seed lot. Gene flow in wheat cropping is currently a concern for certified seed production (Hucl and Matus-Cadiz, 2001). The potential for gene flow is also a component of risk assessment for all genetically engineered (GE) crops (Andow and Zwahlen, 2006). No GE wheat varieties have been commercialized to date, but information about the two sources of gene flow in wheat is relevant for developing appropriate seed production practices.

Little information is available on seed-mediated gene flow occurring during wheat seed production. The origin of off-types in pedigreed wheat seed production has been evaluated by Appleyard et al. (1979) and Hucl et al. (2004), but these studies did not address production practices that might impact the magnitude of seed-mediated gene flow. Production practices that could potentially influence seed-mediated gene flow may include volunteer plants from previous crops and mechanical mixture during harvesting and seed cleaning. The current certified seed standard for allowable presence of a

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detectable off-type variety in a Colorado certified wheat seed lot is 1 in 1000, or 0.1% (Colorado Seed Growers Association, 2003), but no standard exists for purity of farm-saved seed. Approximately 24% of grain production in Colorado is from fields planted to certified seed, indicating that most wheat in Colorado is produced from farm-saved seed (B. Erker, Colorado Seed Growers Association, personal communication, 2006). Seed-mediated gene flow from seed production practices, whether certified or farm-saved, may contribute to adventitious presence of undesired seed in wheat seed or grain lots.

Imidazolinone-resistant (IR) wheat is useful for managing jointed goatgrass (*Aegilops cylindrica* Host) in wheat (Ball et al., 1999). Because wheat and jointed goatgrass share the D genome, wheat pollen can fertilize jointed goatgrass (Hanson et al., 2005) and the resulting hybrid can serve as a bridge to transfer herbicide resistance to jointed goatgrass (Wang et al., 2001). Resistance management plans have been developed to reduce the risk of gene introgression from IR wheat to jointed goatgrass when IR wheat is planted intentionally (Tan et al., 2005), and one component of resistance management is a prohibition on saving IR wheat seed for replanting. Adventitious presence of IR wheat in either farm-saved or certified non-IR wheat seed lots due to seed-mediated gene flow could increase the risk of gene introgression from wheat to jointed goatgrass.

The release of the nontransgenic IR hard red winter wheat cultivar 'Above' (Haley et al., 2003) provided an ideal candidate trait for measuring seed-mediated gene flow. The herbicide resistance trait is a single-gene partially dominant trait on chromosome 6DL resulting from a chemically induced mutation in the acetolactate synthase (ALS; EC 4.1.3.18) gene (Tan et al., 2005). This variety was released to seed producers in 2001 and to commercial growers in 2002. The possibility exists that seed-mediated gene flow may occur during seed production. The magnitude of seed-mediated gene flow may depend on IR wheat production history as well as seed production class (certified or farm-saved). The objective of this study was to compare varietal purity of central western Great Plains certified and farm-saved seed production systems based on the probability of adventitious IR wheat occurrence in non-IR wheat seed lots.

MATERIALS AND METHODS

Sample Collection

Candidate certified seed producers were identified through the Colorado Seed Growers Association. Candidate producers for farm-saved seed samples were identified through Colorado State University Cooperative Extension agents and personal contacts. Producers willing to participate in the study were classified by whether they had produced the IR wheat cultivar Above during the 2005 growing season or any previous growing season. Information about previous crop rotations, harvesting methods, and seed cleaning was obtained through interviews with participating producers.

Each seed sample was from a distinct seed lot based on variety and production classification. Certified seed samples of 160 g were taken from representative seed lot samples kept in storage in the Colorado Seed Testing Laboratory (Fort Collins, CO). These samples had been harvested, cleaned, and collected according to Colorado certified seed protocols. Cooperating farmers and extension agents obtained representative samples of farm-saved seed used for planting. These samples ranged in size from 1 to 2 kg.

Fifty samples of seed produced in 2004 or 2005 were collected from five certified seed producers who had grown Above both years. Seventeen samples of seed produced in 2005 were collected from five certified seed producers who had never grown Above. Twelve samples of seed produced in 2005 were collected from eight producers who saved seed of non-IR varieties grown on their farms and also grew Above. Finally, 13 samples of seed produced in 2005 were collected from 10 producers who saved seed of non-IR varieties grown on their farms and had never grown any IR wheat.

Screening Method Development

The imidazolinone resistance trait of Above wheat provided a selectable marker that was used to screen large samples. A seed-soaking method was used to determine the frequency of herbicide resistant seed in each sample. A dose response experiment was conducted using Above and the non-IR wheat cultivar 'Ike' (Martin et al., 1995) to test the seed-soaking method. For this test, 3-g seed samples were incubated on a shaker table at 50 rotations min^{-1} for 24 h in solutions of 0, 1, 5, 10, 20, 50, 100, 200, and 500 μM of imazamox [(RS)-2-(4-isopropyl-4-methyl-5-oxo-2-imidazolin-2-yl)-5-methoxymethylnicotinic acid] herbicide. To ensure that the method would be able to select heterozygote as well as homozygote-resistant individuals, heterozygote seed from controlled crosses of Above by non-IR wheat were also evaluated. As a limited number of heterozygote seed were available only two concentrations of imazamox, 20 and 50 μM, were tested.

The seeds were planted into 15- by 10-cm pots containing commercial potting media (Sunshine Mix no. 3, SunGro Horticulture, Bellevue, WA). A further 2 cm of potting media was placed over the seeds. The pots were placed in a greenhouse under natural light conditions supplemented with 400 W sodium halide lamps to provide a 14-h daylength and watered as required. Daytime temperatures were approximately 24°C and nighttime temperatures were approximately 18°C. The experiments were assessed 10 d after sowing and plants that emerged from the potting media and reached the two-leaf stage were considered to have emerged. The experiments were repeated twice with three replicates in each experiment.

Sample Screening

A total of 150 g of seed from each production class sample was split into two 75-g portions, placed in 1-L glass jars, and mixed with 250 mL of a 25 μM solution of imazamox to test for presence of IR seed. The samples were thoroughly mixed and placed onto a shaker table at 50 rotations min^{-1} for 24 h. Each seed sample was then spread on the surface of potting media in a 30- by 60-cm flat and covered with 2 cm of potting media. The flats were placed in a greenhouse under the same light

and temperature conditions used in the method development. Plants were watered daily.

A subsample of 3 g was taken from each sample to estimate expected germination. These subsamples were soaked in 10 mL of distilled water for 24 h and then planted in potting mix and placed in the same greenhouse conditions as described above. A count of total emerged plants was taken 2 wk after planting. The number of emerged plants from the 3-g subsample was used to calculate the expected germination from the 150-g sample used for screening.

A foliar application of imazamox was applied to samples 10 to 14 d after emergence to eliminate any susceptible plants that escaped imazamox treatment during seed soaking. The application rate was 35 g ha^{-1} imazamox with 0.25% (v/v) nonionic surfactant (Activator 90, Loveland Industries Inc., Greeley, CO) and 1.0% (v/v) urea ammonium nitrate in a pressured spray chamber calibrated to deliver 187 L ha^{-1} at 206 kPa. Seedlings were clipped with electric shears 2 d after spraying to remove the top leaves and plants that regrew were considered resistant. A total count of resistant plants from each sample was taken and survivors were transplanted. The number of resistant plants was expressed as a percentage of the expected number of germinating plants.

Leaf tissue samples were taken from surviving plants for genetic analysis. A subsample of five surviving plants was taken if a sample had more than 10 survivors. Using proprietary polymerase chain reaction (PCR) based protocols and primers from the BASF Corporation (Research Park Triangle, NC), survivors were tested for presence of the same mutation in the ALS gene sequence as in the cultivar Above. The PCR protocol determined whether the surviving plants were homozygous or heterozygous for the trait.

Data Analysis

The dose response data were analyzed by log-logistic analysis (Seefeldt et al., 1995) using Prism (GraphPad Software, San Diego, CA). The seed-lot data were analyzed as a two-factor generalized linear mixed model using Proc GLIMMIX (SAS Institute, 2005). Occurrence of imidazolinone resistant seed in a sample was modeled as a binomial random variable. The logit of the binomial probability was modeled as fixed effects of production class (certified, farm-saved) and previous IR production (yes or no) and an interaction. Grower and sample nested within grower were normal random effects nested within production class and IR history. One-sided tests of main effects were used due to the assumption that farm-saved production and a positive history of IR production would not decrease the probability of occurrence. Since production practices including field rotations may vary across years for a grower, samples collected from the same grower in two different years were treated as samples from different growers.

RESULTS

Screening Method Development

In the dose response study, there was no significant difference between runs of the experiment, so data were pooled across runs. The IR wheat cultivar Above emerged successfully at all concentrations of imazamox up to 100 μM (Fig. 1). The non-IR cultivar Ike was susceptible to imazamox, with less than 2% of seedlings emerging at concentrations higher than 20 μM. Emerged seedlings of Ike indicated that the screening method eliminated most susceptible seedlings but a small fraction did not imbibe sufficient amounts of imazamox to inhibit germination. An additional foliar imazamox application was necessary to eliminate all false positives. The LD_{50} for Above was 462 μM, whereas that of Ike was 6.7 μM. Emergence of the heterozygotes was 100% at both imazamox concentrations tested (data not shown); however, the heterozygotes were stunted at 50 μM imazamox. Therefore, 25 μM imazamox was chosen as the concentration for screening the seed lots for resistance.

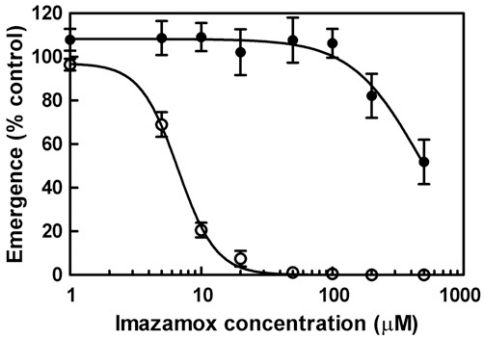

Figure 1. Response of a non-imidazolinone-resistant ('Ike', ○) and an imidazolinone-resistant ('Above', ●) wheat (*Triticum aestivum* L.) cultivar to 24-h seed soaking in various concentrations of imazamox herbicide. Data are means with standard error bars of six replicates across two experiments. Lines are log-logistic fits to the data and are $y = 100/\{1 + e^{[(0.824 - \log x)^* - 6.915)]}\}$ for Ike and $y = 108.3/\{1 + e^{[(2.665 - \log x)^* - 3.829)]}\}$ for Above.

Sample Screening

The total emerged plants evaluated for each sample ranged from 4000 to 7000. Imidazolinone-resistant seeds were detected in samples from growers of each producer type and with each IR production history (Table 1). Detected levels of IR seed ranged from a low of 0.01% to a high of 11.28%. One sample from certified IR Grower 5 (Table 1) considerably exceeded the 0.1% certified seed threshold for presence of other varieties with 4.2% IR wheat in a non-IR seed lot. This seed was produced in a field that was in tilled fallow the previous season and planted to Above wheat two seasons previously. One certified non-IR producer (Grower 9, Table 1) had detectable IR seed in one sample. This producer commercially cleans wheat seed and cleaned seed for Grower 5 in 2005, which may explain the adventitious presence of IR wheat seed in one sample.

Three farm-saved samples from Growers 12 and 15 were higher than 0.1% resistant (Table 1). Grower 15 produced seed in a field that had been planted to Above wheat two seasons previously. Grower 12 had not previously produced Above in the same field as the sampled seed lots, but did harvest Above before harvesting the non-IR varieties

Table 1. Percentage imidazolinone resistant (IR) wheat (*Triticum aestivum* L.) seed detected in non-IR wheat seed lots from certified and farm-saved seed production.

Producer type	IR history†	Year	Grower ID	No. samples	% IR seed‡
Certified	Yes	2005	1	3	0.03
			2	7	0
			3	7	0
			4	7	0
			5	5	4.2, *0.02*
		2004	1	4	0
			2	6	0
			3	4	0
			4	4	0
			5	3	0
Certified	No	2005	6	6	0
			7	1	0
			8	2	0
			9	6	0.02
			10	2	0
Farm-saved	Yes	2005	11	2	0.03, *0.01*
			12	3	11.28, 1.69
			13	2	0.02
			14	1	0
			15	1	0.25
			16	1	0
			17	1	0
			18	1	0
Farm-saved	No	2005	19	4	0.02
			20	1	0
			21	1	0
			22	1	0
			23	1	0
			24	1	0
			25	1	0
			26	1	0
			27	1	0.02
			28	1	0

†Imidazolinone resistant (IR) wheat production history, indicating whether a producer grew the IR wheat cultivar 'Above' (Yes) or had never grown Above (No).

‡Percentage of seeds screened that were confirmed IR where % IR seed = number of confirmed IR seeds/total emerged seeds × 100. Each value indicates a separate sample for which IR was detected; any remaining samples from a grower with no resistance detected are not listed. Underlined values indicate that plants were confirmed as homozygous for the IR trait and values in italics indicate that one or more IR plants were heterozygous for the IR trait.

with the same equipment. The high levels of IR seed in these samples may be due to mechanical mixture at harvest, or may reflect adventitious IR seed from previous years in which Above was grown and non-IR seed was saved.

Results from Proc GLIMMIX indicated that variation due to production class (certified, farm-saved) was significant at $\alpha = 0.05$ (one-sided $P = 0.0435$). Variation due to IR history was significant at $\alpha = 0.1$ (one-sided $P = 0.0799$). The interaction was not significant. The estimated proportion of IR seed in the median sample of the median producer was less for certified (8.4×10^{-6}) compared to farm-saved (66×10^{-6}), and less for negative IR history (10×10^{-6}) compared with positive (54×10^{-6}) (Table 2). On average, both producer types and both IR production histories had an estimated proportion of adventitious presence that is considerably lower than the 0.1% threshold for certified seed. However, these estimates do not indicate that levels of adventitious presence substantially above the 0.1% threshold will not occur, as some samples did exceed the threshold (Table 1).

Based on the PCR results, nearly all of the 30 resistant plants tested (93%) were homozygous for the resistance mutation; the remaining 7% (2 plants) were heterozygous. All plants tested carried the mutant resistance allele. Plants heterozygous for the mutation, as determined by the PCR test, were found in one certified IR producer sample and in one farm-saved IR producer sample, with the majority of plants in both samples being homozygous. The sample containing a heterozygous plant from Grower 5 (Table 1) was produced in a field that bordered Above wheat, so pollen-mediated gene flow is a reasonable explanation. All resistant plants from certified non-IR producer samples and farm-saved non-IR producer samples were homozygous (Table 1).

DISCUSSION

This study provides the first report of the frequency and magnitude of adventitious presence of unintended wheat seed during seed production in the central western Great Plains growing region. These results indicate that seed-mediated gene flow is occurring in both certified and farm-saved seed production. Because most of the resistant plants detected in non-IR seed lots were homozygous, seed mixture is a more likely explanation for the source of gene flow than pollen drift during the growing season, which would have produced heterozygotes in the generation after cross-pollination occurred. Although the detected resistance could have developed through spontaneous mutation, this is unlikely because the PCR protocol specifically targeted the mutant ALS allele carried by Above wheat and all tested resistant plants contained this allele.

Analysis of the data indicates that certified grower seed lots are less likely to have adventitious presence than farm-saved grower seed lots. These data are also suggestive that adventitious presence of a particular variety is more likely when a grower has a history of producing that variety. The implication is that combining the higher-probability production class (farm-saved) with the higher-probability variety history (positive) produces the highest adventitious presence.

These results are based on adventitious presence of a nontransgenic wheat cultivar with no marketing restrictions, but they have implications for relevant policies regarding production of wheat seed with genetic traits that

Table 2. Estimated proportion of adventitious imidazolinone resistant (IR) wheat (*Triticum aestivum* L.) seed in the median sample of the median producer in each class.

Main effect	*P*	Level	Proportion IR seed ($\times 10^{-6}$)	SE ($\times 10^{-6}$)
Production class	0.0435	Certified	8.4	7.3
		Farm-saved	66.0	50.0
IR history	0.0799	Yes	54.0	34.0
		No	10.0	9.9

may be unacceptable in certain markets. A similar study using certified canola (*Brassica napus* L.) seed lots in Canada found that herbicide resistance traits could be detected in lots of nonresistant cultivars (Friesen et al., 2003) and concluded that variety purity could not be maintained at a 99.75% level. The results of our study are consistent with the canola study and indicate that a "zero-tolerance" policy for seed lot purity is unachievable under current wheat seed production practices.

A potential source of seed-mediated gene flow is volunteer wheat plants from previous crops. Volunteer wheat seed can survive at least 16 mo in soil (Anderson and Soper, 2003). Samples from two growers had levels of IR wheat higher than 0.1% and were produced in fields were IR wheat had been grown 2 yr previously. The Colorado seed certification standards establish land requirements for small grains (Colorado Seed Growers Association, 2003). A certified seed crop must be planted on land that did not grow the same crop in the previous season, while registered seed must be planted on land that did not grow the same crop in the previous two seasons. A two-season restriction is required when a white wheat follows a red wheat or vice versa. These requirements recognize the importance of minimizing volunteer wheat to produce a pure seed lot. The two examples of detectable carryover of IR wheat from a crop grown 2 yr previously indicate that a two-season restriction may not always be adequate.

Mechanical mixture during harvesting and seed cleaning was apparently associated with IR wheat presence from a farm-saved source. Colorado seed certification standards establish requirements for thorough cleaning of equipment before harvesting and for seed cleaning that would not apply to farm-saved seed production (Colorado Seed Growers Association, 2003). Based on the results of this study, requirements for equipment cleaning appear to be critical for reducing the frequency of adventitious seed presence. The level of adventitious presence associated solely with seed cleaning was less than 0.05% and is likely an acceptable level.

The presence of IR wheat in non-IR wheat seed lots provides an unmanaged pollen source for transfer of herbicide resistance to jointed goatgrass. Producers growing IR wheat will likely apply an imidazolinone herbicide during a growing season, achieve good control of jointed goatgrass, and reduce the chance of resistant hybrid formation. Producers planting non-IR seed with adventitious IR seed into a field containing jointed goatgrass would not apply an imidazolinone herbicide, thereby increasing the likelihood of resistant hybrids occurring. Since volunteer IR wheat was detected in seed from certified and farm-saved seed growers, unmanaged volunteer IR wheat may also contribute to gene flow to jointed goatgrass in subsequent wheat cropping years after the use of IR wheat.

In conclusion, adventitious presence of nontransgenic herbicide resistant wheat seed was detected from both certified and farm-saved seed producers. Based on grower interviews, higher levels of adventitious seed presence were associated with volunteer plants from previous crops of the resistant cultivar and mechanical mixture during harvesting. Seed cleaning was associated with much lower levels of adventitious presence. Production practices for certified seed address these factors and may need to be strengthened if more stringent purity criteria are adopted. This information is important for risk assessment and policy development for potential commercial release of transgenic wheat varieties.

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